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INTRODUCTION

This publication is a summary of the following communications, referred to by Roman numerals as follows:

I. Ljunger, C. 1962. Introductory investigations of ions and thermal resistance. — Physiol. Plant. 15:148—160.

II. Ljunger, C. 1963. Ions and thermal resistance. Studies on growth. — Ibid. 16:121—131.

III. Ljunger, C. & Larsson, K. 1973. On the nature of the heat stabilizing effect of inorganic ions on Escherichia coli. — Ibid. 28:299—303.

IV. Ljunger, C. 1968. The minimum growth temperature of obligately thermophilic bacteria as influenced by inhibitors in complex growth media. — Ibid. 21:26—34.

V. Ljunger, C. 1970. On the nature of the heat resistance of thermophilic bacteria. — Ibid. 23:351—364.

VI. Ljunger, C. 1973. Further investigations on the nature of the heat resistance of thermophilic bacteria. — Ibid. 28(3):415—418.

VII. Ljunger, C. & Edebo, M. 1972. The influence of inorganic ions on the heat stability of a thermophilic bacteriophage. — Ibid. 27(2):182—186.

VIII. Ledebo, I. & Ljunger, C. 1973. Permeability of bacteria to inorganic cations. A comparison between a psychrophilic Achromobacter strain and Escherichia coli. — Ibid. (in press).

Regardless of the dates of publication the articles should be studied in the arranged order, since it indicates the actual sequence in which the experiments were performed.

THE PRINCIPAL CONTENTS OF THE INDIVIDUAL ARTICLES

I. During an investigation of the occurrence of coliform bacteria on vegetables a number of strains were isolated which showed á delayed production of gas when grown in lactose nutrient broth at 37°C. Some strains did not produce gas at all under these conditions. However, when ox bile was included in the lactose broth, most of these isolates produced gas within 24 hours' incubation at 37°C. Ox bile is used in the preparation of selective media for the isolation of coliform bacteria because of its inhibitory effect on the developement of gram-positive bacteria. The above-mentioned effect of ox bile on the gas production from lactose at 37°C was caused by some inorganic component(s) of the bile, since it was also obtained when incinerated ox bile was added to the lactose broth. Furthermore, it was found that the ash of ox bile could be replaced by the chlorides of magnesium, calcium, barium, strontium, sodium and potassium. When present in certain concentrations all of these salts caused gas production from lactose within 24 hours' incubation at 37°C. Since the tested coliform strains fermented lactose with gas production within 24 hours at 30°C, it was assumed that the ions stabilized enzymes involved in the gas production against thermal inactivation at 37°C. This assumption was supported by the fact that the effect of the ions was nonspecific inasmuch as the ordinary maximum temperatures of several bacterial activities, including growth, were increased by the addition of inorganic ions to the culture medium.

II. The influence of magnesium, calcium, sodium and potassium chloride on the growth of *Escherichia coli* at various temperatures was examined. It was observed that certain ion concentrations had a stimulating effect on growth at temperatures close to the ordinary maximum growth temperature of this organism but were inhibitory to growth at lower temperatures. This would imply that the assumed stabilizing effect of the ions on enzymes against heat inactivation was accompanied by a reduced enzyme activity. Accordingly, the observed effects of the ions on growth of *E. coli* at various temperatures could be explained in the following way. At low temperatures thermal inactivation of enzymes is of relatively little importance and thus the inhibitory effect of the ions on the activity of enzymes involved in the growth process is pronounced. At high temperatures, however, the thermal inactivation of enzymes is

extensive and the stabilizing effect of the ions manifests itself as a stimulation of growth as compared with growth without added ions.

III. It seemed likely that information on the mode of action of the inorganic ions would be obtained by comparing the amounts of the different ions that have the same effect on some bacterial activity at an elevated temperature. The reducing effect of added ions on the growth lag of *Escherichia coli* at 45°C observed in a previous investigation (II) was used in a quantitative comparison between the effects of the ions, because it was assumed that changes in the composition of the growth medium were minimal during the lag phase of growth. 45°C is very close to the maximum growth temperature of *E. coli* under ordinary growth conditions, and the lag-reducing effect of the ions was probably the result of an increased thermal stability of enzymes involved in synthesizing reactions during this phase of growth. The results of the experiments showed that equivalent amounts of the ions had the same lag-reducing effect. Hence this effect was not dependent on the number of ions but on the number of ion charges. This circumstance supported the assumption that the ions combine with negatively charged groups of enzyme proteins.

Many species of gram-negative bacteria are plasmolyzed by hypertonic salt solutions. The plasmolysed cells scatter more light, which can be registered with photometric methods. If the solute penetrates the cytoplasmic membrane, deplasmolysis will occur and the light-scattering of the cells returns to its original value. Using this technique it was found that cells of *E. coli* suspended in peptone broth were permeable to magnesium, calcium, sodium and potassium ions.

IV. The observation that the stimulating effect of the ions on growth of mesophilic bacteria at high temperatures was associated with an inhibitory effect on growth at low temperatures (II), led to be assumption that the extremely high maximum growth temperatures of obligately thermophilic bacteria as well as their remarkably high minimum growth temperatures might be dependent on the content of inorganic ions in the growth medium. The definition by Cameron and Esty (1926) of obligately thermophilic bacteria as being able to grow at or above 55°C but not at 37°C is still in common use. In attempts to decrease the minimum growth temperatures of various obligately thermophilic strains of *Bacillus stearothermophilus* by reducing the electrolyte content of commonly-used complex growth media it was found that these bacteria

were capable of vigorous growth at 37°C. This ability was, however, obviously not caused by the removal of electrolytes from the growth media but by the removal of growth inhibitors of organic nature. These inhibitors could be removed from the various growth media with the same methods, i. e. extraction with chloroform or adsorption on ion exchange resins or starch. This fact suggests that they were related or identical. Some observations indicated that they might consist of bile acids. The results of this investigation indicate that the established definition of obligately thermophilic bacteria needs a modification.

V. From results obtained in studies on the effect of inorganic ions on the thermal resistance of mesophilic bacteria (I, II) it seemed possible that the ability of thermophilic bacteria to resist high tempartures might be related to the environmental electrolyte concentration. When incubated at 55°C in a nutrient-free buffer solution thermophilic bacteria rapidly die (Allen 1950). In a series of experiments components of a medium permitting excellent growth of *B. stearothermophilus* at 55°C were added to tris-HCl buffer and the survival of this bacterium at 55°C in the various buffer solutions was determined. The results of these experiments showed that the heat resistance of the cells was dependent on the presence of calcium ions in the surrounding medium. Besides calcium ions, potassium and phosphate ions and glucose or some other energy source were required. These requirements indicated that the heat stability of thermophilic bacteria is attributable to an active transport of calcium ions from the environment into the cells. The active uptake of divalent cations by yeast cells is likewise found to be dependent on the presence of phosphate and glucose and the rate of uptake is markedly increased in the presence of potassium ions (Rothstein 1961).

VI. In a previous investigation of the heat resistance of thermophilic bacteria (V) it was observed that when the cells of a growing culture of *B. stearothermophilus* were harvested and then washed with tris buffer, the content of calcium ions in the buffer exceeded the amount expected. Furthermore, a fraction of the cells rapidly died when they were suspended in the buffer. Since the cells were supposed to take up calcium actively from the growth medium, it seemed possible that they were osmotically shocked when suspended in the buffer. To test this possibility *B. stearothermophilus* was grown in the presence of various amounts of calcium chloride. When the cells were subsequently suspended in tris

buffer, it was found that the fraction dying in the buffer increased with increasing concentration of calcium ions in the growth medium. The reduction in the number of viable cells was associated with a release of calcium ions and organic material from the bacteria. When, instead of calcium chloride, magnesium, sodium or potassium chloride was added to the growth medium no reduction in the number of viable cells was obtained after transfer to tris buffer.

The results obtained in this investigation indicated that calcium was taken up from the growth medium and accumulated inside the cells. When these were subsequently suspended in tris buffer, their osmotic barrier was damaged, which caused a leakage of intracellular material from the cells. To be osmotically active calcium must exist in free form and not be bound to macromolecules in the cells.

VII. Since calcium ions were found to be essential for the heat resistance of thermophilic bacteria it was of interest to examine if calcium also had an influence on the heat stability of thermophilic bacteriophages. A thermophilic *Bacillus* strain and a phage active against it were isolated from soil. Heat inactivation experiments were carried out with phages suspended in tris buffer supplemented with various amounts of calcium, magnesium, sodium and potassium chloride. The phage suspensions were heated at 65°C for two hours. Calcium and magnesium ions were found to stabilize the phage particles against heat inactivation, whereas comparable concentrations of sodium and potassium ions did not. Furthermore, equimolar concentrations of calcium and magnesium had the same stabilizing effect.

VIII. Previously obtained results (II, V) suggested that the temperature characteristics of psychrophilic bacteria depend on the exclusion of inorganic ions from the cells of these organisms. To test this possibility the permeability of a psychrophilic *Achromobacter* strain to magnesium, calcium, sodium and potassium chloride was investigated. Cells of the psychrophile were plasmolyzed by suspending them in hypertonic solutions of the various salts in peptone broth. The plasmolysis was associated with an increase in the light-scattering capacity of the cells, which was registered with a nephelometer. A subsequent deplasmolysis resulted in a decrease in the scattered light. The data obtained indicated that the psychrophilic cells were completely permeable to sodium and potassium ions, whereas magnesium and calcium ions were

excluded to a considerable extent. When cells of *E. coli* were tested under the same condtions, they were found to be permeable to all four cations. This is in accordance with an earlier observation (III).

GENERAL DISCUSSION

It was found that the ordinary maximum temperature for gas production from lactose by some strains of coliform bacteria could be increased by the addition of various inorganic ions to the growth medium (I). This observation led to the assumption that the ions had a stabilizing effect on enzymes involved in the gas production against thermal inactivation. In another study the influence of added inorganic ions on the growth of *E. coli* at various temperatures was examined (II). The results of that investigation suggested that the stabilizing effect of the ions on enzymes is associated with a reduced enzyme activity. The data obtained in a following investigation (III) indicated that the ions act by means of their charges, since equivalent amounts of the tested ions had the same heat stabilizing effect on enzymes.

The interactions between inorganic ions and proteins are complex, but it seems reasonable to assume that monovalent cations may increase the heat stability of proteins by associating to negatively charged groups of the protein molecules and thereby reduce their hydration. Divalent cations may act in the same way, or they may form stabilizing cross-links between negative groups of the proteins. Both modes of action may be of importance. A decrease in the hydration and a blocking of certain groups of enzymes should give rise to a reduction in their activity. Thus the heat stabilizing effect of the ions on enzymes should be accompanied by a reduced enzyme activity. At high temperatures the former effect is manifested; at low temperatures, the latter.

The ability of thermophilic bacteria to grow at high temperatures requires that their enzymes function at these temperatures. Thus, these bacteria must posses either heat stable enzymes or the ability of a rapid resynthesis of inactivated enzymes. Various cell components of thermophilic bacteria have been reported to be heat stable, among them flagella (Koffler 1957), ribosomes and ribosomal RNA (Saunders and Campbell 1966, Friedman, Axel and Weinstein 1967), DNA (Stenesh, Roe and Snyder 1968) and several enzymes (for references see V and VI). Almost all these reports deal with crude or partly purified preparations. Therefore they do not establish to what extent the heat stability

is caused by an inherent heat stability of the studied cell components or by associated stabilizing factors. Manning and Campbell (1961) described a purified and crystallized α-amylase from *Bacillus stearothermophilus* as extremely resistant to heat inactivation. The purity of this enzyme preparation is often quoted to exclude the possibility that its heat stability was caused by the presence of some stabilizing factor. However, the crystallized enzyme was also reported to contain firmly bound calcium. Recently the presence of firmly bound calcium in a heat stable protease from a thermophilic *Bacillus* species has been established by means of a comprehensive X-ray diffraction analysis (Matthews et al. 1972). The bound calcium seemed to be essential for the heat stability but not for the activity of this enzyme.

Regardless of how many heat stable, or heat stabilized, structures one may isolate from thermophilic bacteria, this is of no decisive importance for the explanation of thermophilic growth. The growing cell is obviously no more heat resistant than the most heat labile of its essential enzymes. Intracellular enzymes of thermophilic bacteria that are easily inactivated by heat in cell-free preparations, but are heat stable in the intact cell, are known (Allen 1950, Militzer and Burns 1954). Furthermore, the fact that thermophilic bacteria lose their heat resistance in nutrient-free buffer solutions (Allen 1950, V) is incompatible with the view that this resistance is caused by inherently heat stable cell components.

The experimental data obtained in studies on thermophilic bacteria (V, VI) strongly indicate that the survival and functioning of these organisms at high temperatures depend on the maintenance of a high intracellular concentration of free calcium ions. This is possible only when the surrounding medium besides calcium also contains phosphate, potassium and a source of energy. The uptake of calcium is probably performed by a transport system with a selective affinity for that ion.

The literature on thermophilic bacteriophages has been reviewed by Farrell and Campbell (1969). They stated that the evidence so far available favours the view that the factors responsible for the heat stability of thermophilic phages reside in some as yet unknown arrangement of the coat protein. The proteins of thermophilic phages should have approximately the same heat stability characteristics as the proteins of their host bacteria. When the influence of inorganic ions on the heat stability of thermophilic phages was examined, it was found that calcium and magnesium ions in equimolar concentrations had the same marked stabilizing effect, while sodium and potassium ions were without

effect (VII). The observation that magnesium was equally effective as calcium in the stabilization of the phages but did not influence the heat resistance of thermophilic bacteria is most easily explained by a selective uptake of calcium by the bacteria.

The high content of calcium in the bacterial endospore is known to be essential for its extremely high heat resistance. The spore has practically no metabolic activity. Obligately thermophilic bacteria actively accumulate calcium inside the cells (V, VI). They are able to grow at high temperatures but have also a remarkable high minimum temperature of growth. The growth of mesophilic bacteria at temperatures close to their maximum growth temperatures can be improved by the addition of inorganic ions to the growth medium (I, II, III). The same addition has an inhibitory effect on mesophilic growth at low temperatures. Psychrophilic bacteria have comparatively low maximum growth temperatures but are able to grow at temperatures around 0°C. It seems reasonable to assume that the temperature characteristics of these bacteria depend on some mechanism for the maintenance of a low intracellular content of inorganic ions. The results of an introductory investigation of this subject (VIII) showed that calcium and magnesium ions to a considerable extent were excluded from the cell interior of a psychrophilic bacterium whereas sodium and potassium ions penetrated the cell.

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